

# Menthol Suppresses Nicotinic Acetylcholine Receptor Functioning in Sensory Neurons via Allosteric Modulation

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## Abstract

In this study, we have investigated how the function of native and recombinant nicotinic acetylcholine receptors (nAChRs) is modulated by the monoterpenoid alcohol from peppermint (–) menthol. In trigeminal neurons (TG), we found that nicotine (75  $\mu$ M)-activated whole-cell currents through nAChRs were reversibly reduced by menthol in a concentration-dependent manner with an  $IC_{50}$  of 111  $\mu$ M. To analyze the mechanism underlying menthol's action in more detail, we used single channel and whole-cell recordings from recombinant human  $\alpha 4\beta 2$  nAChR expressed in HEK tsA201 cells. Here, we found a shortening of channel open time and a prolongation of channel closed time, and an increase in single channel amplitude leading in summary to a reduction in single channel current. Furthermore, menthol did not affect nicotine's  $EC_{50}$  value for currents through recombinant human  $\alpha 4\beta 2$  nAChRs but caused a significant reduction in nicotine's efficacy. Taken together, these findings indicate that menthol is a negative allosteric modulator of nAChRs.

**Key words:** allosteric modulation, electrophysiology, HEK tsA201 cells, human  $\alpha 4\beta 2$  nicotinic receptor, menthol, nicotine, rat, single channel recording, trigeminal ganglion neuron

## Introduction

Menthol is a fragrant monoterpenoid alcohol derived from peppermint (*Mentha x piperita*) oil. Its cooling sensation when topically applied to the skin or mucous membranes of the airways (Eccles 1994) is a well-liked effect, which is used in many oral health care products, cosmetics food products, and tobacco. A range of somatosensory sensations have been reported to be modulated or activated by menthol and include warmth, burning, irritating, or painful sensations (Green 1992; Cliff and Green 1994; Namer et al. 2005).

### Menthol acts on a variety of different membrane receptors

Recent studies showed that menthol is a specific activator of TRPM8, a member of the TRP superfamily that acts as thermosensor in the somatosensory system (Damann et al. 2008; Talavera et al. 2008). This receptor is expressed in a subset of trigeminal and dorsal root neurons (McKemy et al. 2002; Peier et al. 2002). Activation of TRPM8 by cold or menthol results in an increase in intracellular  $Ca^{2+}$  concentration (Okazawa et al. 2000; McKemy et al. 2002; Peier et al. 2002; Reid et al. 2002), and menthol-induced release from intracellular  $Ca^{2+}$  stores has been shown to enhance neurotransmission at sensory synapses (Tsuzuki et al. 2004).

Another member of the TRP family, TRPA1, is activated by menthol at lower concentrations but inhibited by higher concentration of menthol (Karashima et al. 2007). The inhibitory effect appears to be species specific as this was observed in mouse but not in human TRPA1; but see Willis et al. (2011). TRPA1 is usually involved in signaling induced by irritant and inflammatory substances (Bandell et al. 2004; Jordt et al. 2004; Bautista et al. 2006). This receptor is expressed in a subset of trigeminal and dorsal root neurons where TRPM8 seems to be absent (Story et al. 2003; Kobayashi et al. 2005).

Other ion channels which are affected by menthol are ionotropic receptors in the mammalian central nervous system (CNS) that mediate inhibitory neurotransmission. On human  $\gamma$ -amino butyric acid type A ( $GABA_A$ ) and glycine receptors, menthol isomers act as potent positive modulators (Hall et al. 2004). These receptors are recognized as important targets for modulation by sedative, anxiolytic, and general anesthetic agents (Franks and Lieb 1994; Krasowski and Harrison 1999).

### Menthol effects on nicotine receptors in sensory neurons

Most recently, it has been shown that menthol acts as a broadband counterirritant against inhaled cigarette smoke

irritants such as acrolein, acetic acid, or cyclohexanone at the respiratory epithelium in mouse. It has been suggested that its counterirritant effect is due the activation of TRPM8 receptors but not TRPA1 receptors (Willis et al. 2011). Nicotine which also acts as an important irritant in cigarette smoke has not been evaluated in this study. Previous psychophysical studies showed that nicotine elicits burning or stinging pain sensation on oral or nasal mucosa (Hummel et al. 1992; Dessirier et al. 1997; Dessirier et al. 1999), and these sensations are thought to involve activation of nicotinic acetylcholine receptors (nAChRs) expressed in the sensory fibers innervating these tissues (Alimohammadi and Silver 2000). Interaction between menthol and nAChRs on human sensory perception was studied by Dessirier et al. (2001), who showed that nicotine-induced irritation on the participants tongue was significantly reduced by menthol pretreatment (cross-desensitization), however, the underlying mechanism has not been determined. The possibility exists that menthols broadband counterirritant action as described by Willis et al. (2011) also affects nAChRs. Alternatively, menthol could directly affect nAChRs to downregulate their function.

### Nicotinic acetylcholine receptors

nAChRs are expressed in the CNS and in several nonneural tissues and are encoded by 9 alpha ( $\alpha_2$ – $\alpha_{10}$ ) and 3 beta ( $\beta_2$ – $\beta_4$ ) subunit genes (Le Novère et al. 2002; Hogg and Bertrand 2004; Gotti et al. 2006). The nAChR family consists of acetylcholine-gated channels that are formed as pentameric arrangement of homogeneous ( $\alpha_7$ ,  $\alpha_8$ ,  $\alpha_9$ ) or heterogeneous (e.g.,  $\alpha_4\beta_2$ ,  $\alpha_2\beta_2$ ) subunit combinations, of which the  $\alpha_4\beta_2$  AchRs represent the major brain subtype. Intraepithelial free nerve endings of the trigeminal nerve innervate the oral and upper respiratory tract and convey sensations from the mucosa (Alimohammadi and Silver 2000) and have been shown to express most genes encoding the major neuronal nAChR subunits ( $\alpha_2$ – $\alpha_7$ ,  $\alpha_9$ , and  $\beta_2$ – $\beta_4$ ) (Liu et al. 1993; Keiger and Walker 2000).

In the present study, we used whole-cell and single channel recordings of currents through nAChR in acutely dissociated trigeminal neurons and human  $\alpha_4\beta_2$  nAChRs stably expressed in HEK tsA201 cells, respectively, to directly analyze the effect of menthol on pharmacological and biophysical properties of nAChRs. We found that nAChR receptor currents were reversibly inhibited by (–) menthol in a concentration-dependent manner. Our results suggest that menthol is a negative allosteric modulator of nAChR proteins.

## Materials and methods

### Cell culture

Trigeminal ganglia were excised from decapitated  $17 \pm 3$ -day-old Wistar rats and incubated  $20 \pm 5$  min in artificial cerebrospinal fluid consisting of (in mM): 124 NaCl, 2.5 KCl,

26 NaHCO<sub>3</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 1.3 MgSO<sub>4</sub>, 2 CaCl<sub>2</sub>, 10 D-glucose, pH 7.35, gassed with Carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) containing collagenase IA (0.7 mg/mL, Sigma-Aldrich), Trypsin (0.3 mg/mL, Roche), DNase (0.01 mg/mL, Roche) at 33 °C. Digestion was stopped by resuspending the tissue in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 units/mL) (Invitrogen). Tissue was triturated mechanically with fire-polished glass pipettes and centrifuged at  $160 \times g$  for 5 min after filtration. Pellet was resuspended with the prior culture medium, and cells were plated on poly-L-lysine-coated glass coverslips and kept in humidified atmosphere (37 °C, 95% air, 5% CO<sub>2</sub>).

The human  $\alpha_4\beta_2$  nAChRs stably transfected in HEK tsA201 cells were kindly provided by J. Lindstrom. Cells were maintained in DMEM with penicillin (100 U/mL), streptomycin (100 µg/mL) (Invitrogen), and 10% fetal bovine serum. Zeocin (0.5 mg/mL) and G-418 (0.6 mg/mL) was used for selection of  $\alpha_4$  and  $\beta_2$  subunit expression, respectively. Cells were plated on poly-L-lysine-coated glass coverslips and used within 24–48 h after plating for recordings.

### Electrophysiology

Cells were examined using whole-cell and cell-attached patch configurations of the patch-clamp technique. Recordings were made with an EPC 9 and Pulse software (both HEKA Electronics), filtered/digitized at 3/10 kHz (4-pole Bessel) for whole cell or at 10/30 kHz (3-pole Bessel) for cell-attached recordings, and stored on hard disk. Recordings were performed from somata of TG neurons (mean  $\pm$  SD [standard deviation],  $33.4 \pm 14.1$  µM,  $n = 124$ ) at room temperature (23–25 °C). Agonist or menthol solutions were prepared daily from stock solution. For whole-cell experiments recording, electrodes were filled with internal solution consisting of (in mM): 130 KCl, 10 NaCl, 10 ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub> (pH 7.35), and filled electrodes had a resistance between 1.5 and 4 MΩ. The external solution contained (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 20 D-glucose, 1.3 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub> (pH 7.35). (–) Menthol, (–) nicotine, or (–) nicotine/(–) menthol were applied in external solution using a fast pressure-application system (DAD-VM Superfusion System, ALA Scientific Instruments). Experiments were conducted only on cells that showed no responses to 500 ms application of bath solution to exclude any possible pressure artifact. Drug solutions were applied for 500 ms or 1 s every 3 min. The normalizing concentration of (–) nicotine (75 µM) was applied several times to each cell during the course of an experiment to check for desensitization and/or rundown. Cells were excluded from analysis if the first 3 control responses showed >15% difference in response amplitude.

Single channel currents from TG neurons were recorded in cell-attached configuration using Sylgard 184 (Dow Corning) coated electrodes fire polished to a resistance of

2.5–5 M $\Omega$ . The bath and pipette solution contained (in mM): 142 KCl, 5.4 NaCl, 10 HEPES, 1.7 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub> (pH 7.3 adjusted with KOH). The pipette solution also contained (–) nicotine 75  $\mu$ M ( $n = 6$ ) or (–) nicotine 75  $\mu$ M/(–) menthol 100  $\mu$ M ( $n = 7$ ) or no drug ( $n = 3$ ). The holding potential for all recordings was –70 mV. Icilin was purchased from Cayman Chemical Co. All other chemicals were obtained from Sigma-Aldrich

### Data analysis

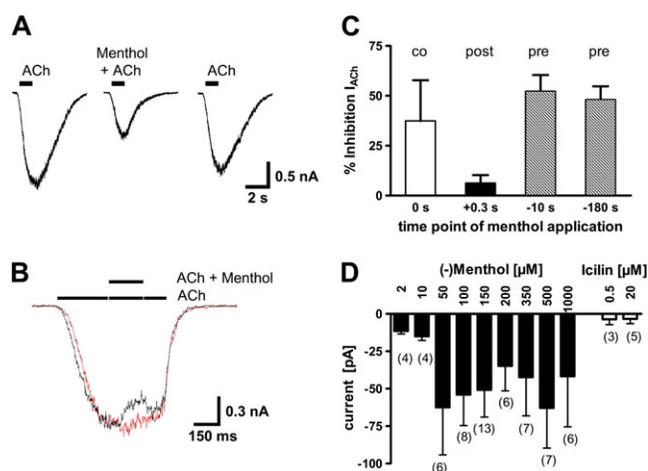
The analysis of whole-cell recordings was carried out offline using PulseFit (HEKA) or IGOR software (Wavemetrics). The concentration–response curves of agonists were constructed in PRISM (GraphPad Software Inc.) by plotting the amplitude of agonist-induced currents (normalized to maximum current amplitude produced by respective agonist for each individual cell) against log agonist concentrations. The EC<sub>50</sub> and Hill slopes were determined by fitting data points to a logistic function. Single channel data were analyzed using QuB software ([www.qub.buffalo.edu](http://www.qub.buffalo.edu)). All of the digitized traces were carefully inspected for artifacts and baseline drift before any quantitative analysis was performed. Only records from patches containing a single active channel were selected for processing and analysis. Periods when the channel was actively gating with homogeneous kinetics were selected from each record using a critical time ( $t_{crit}$ ) of 1 s. Closed intervals longer than  $t_{crit}$  were removed, and the remaining intervals were joined to create an “active-time” record. Idealization of the currents was performed at a bandwidth of 10 kHz using the segmentation  $k$ -means hidden Markov algorithm (Qin 2004) with a C  $\leftrightarrow$  O model (both rate constants = 100 s<sup>–1</sup>) or by a half-amplitude threshold-crossing algorithm after additional low-pass filtering to 3 kHz to obtain single channel open amplitude, open probability, and mean open and close times. Time constants and areas of the various components of the dwell-time distributions were estimated by using maximum-likelihood methods.

For detailed statistical analysis, GraphPad Prism 4.0 (GraphPad) was used. The data in manuscript are presented as mean  $\pm$  SD unless stated otherwise.  $N$  represents number of cells analyzed, and for single channel recordings, it represents the number of events analyzed. The significant difference between groups was assessed by one-way analysis of variance followed by post hoc statistical analysis (Bonferroni or unpaired  $t$ -test).

## Results

### (–)Menthol reversibly inhibits nAChR-induced whole-cell currents in a time- and concentration-dependent manner

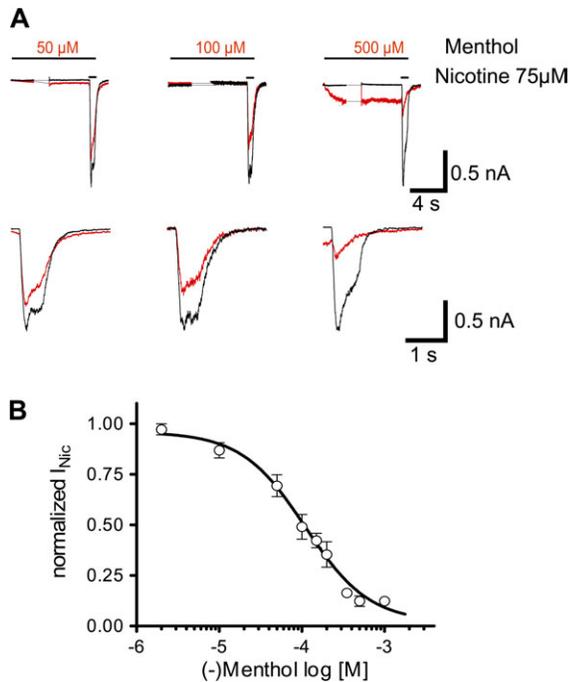
We first examined the effect of (–) menthol (menthol) on whole-cell currents through nAChR in sensory neurons. Figure 1 illustrates individual currents elicited by brief applications of nAChR agonist ACh (EC<sub>50</sub> = 75.7  $\mu$ M, data not



**Figure 1** Application of (–) menthol reduces agonist-induced currents through nAChRs in trigeminal neurons. **(A)** Representative currents recorded from an acutely isolated trigeminal neuron induced by ACh (100  $\mu$ M) under control conditions (left trace), during coapplication ACh and 100  $\mu$ M (–) menthol (middle trace), and after a 3-min wash period (right trace). Drugs were applied for the period indicated by the horizontal bar. Holding potential: –70 mV. **(B)** Currents recorded in response to ACh (100  $\mu$ M, gray trace) or with intermittent 200 ms coapplication of ACh and menthol (each 100  $\mu$ M, black trace). Note the full reversibility of the menthol-induced inhibition upon change to ACh. In control experiments, where ACh instead of menthol was applied there is no alteration in the current kinetic during coapplication visible (gray trace). **(C)** The ACh-induced current inhibition by 100  $\mu$ M menthol depends on the time point of menthol application. Menthol was applied either together with ACh (co, as in Figure 1A), during (post, as in Figure 1B), or before ACh application (pre). The time points are given in seconds with respect to the onset of ACh application. Each bar represents the mean  $\pm$  standard error of the mean (SEM). **(D)** Average membrane currents in trigeminal neurons elicited by 10 s application of menthol or icilin at the indicated concentrations. Each bar represents the mean  $\pm$  SEM, and  $n$  is given in parenthesis above the individual bars. This figure appears in color in the online version of *Chemical Senses*.

shown) or ACh/menthol mixture using a rapid drug application system. When ACh (100  $\mu$ M) was coapplied with menthol (100  $\mu$ M), we observed a fully reversible and significant reduction of the ACh-induced current amplitude ( $37.4 \pm 20.4\%$ ,  $n = 4$ ,  $P < 0.005$ ; Figure 1A) without alterations in current kinetics. Pretreatment of the cell with menthol (100  $\mu$ M) for 10 s prior to the ACh/menthol coapplication caused a reversible and significant reduction of the current amplitude by  $52.3 \pm 8.1\%$  ( $n = 5$ ,  $P < 0.001$ ; Figure 1C; see also Figure 2A). Further increase of the pretreatment period to 180 s resulted in a similar menthol-induced reduction ( $48.1 \pm 6.6\%$ ,  $n = 3$ ,  $P < 0.001$ ; Figure 1C), however, the inhibition was only partially reversible (<60%). Although the menthol pretreatment for 10 or 180 s appeared to increase the degree of inhibition of the ACh-induced current compared inhibition observed with menthol coapplication, this increase was not significant ( $P > 0.1$ ). Similar results were obtained when the bath solution contained 1  $\mu$ M atropine to block muscarinic AChR.

To test whether the interaction of menthol with the nAChR depends on the conformational state of the receptor, we used a protocol where a mixture of ACh/menthol (each at



**Figure 2** (A, upper panel) Nicotine-induced currents (75  $\mu$ M) were elicited following a 10 s application of either control- (black trace) or menthol-containing solution (red trace, used concentration is indicated above each trace). (A, lower panel) The last 3 s of the recordings are shown on an expanded time scale. (B) The concentration–response curve for inhibition of nicotine-induced currents by (–) menthol was constructed from A. Nicotine-induced responses obtained at different menthol concentrations were normalized to control response (75  $\mu$ M nicotine) and plotted against the menthol concentration.  $IC_{50}$  value and Hill slope were obtained by fitting the average data points to a logistic equation (see Materials and methods), and the best fit is represented by the solid line through the data points. The  $IC_{50}$  value for (–) menthol was  $111.4 \pm 2.5 \mu$ M, Hill slope = 1.1. Each data point represents the mean  $\pm$  standard error of the mean of 6–13 cells.

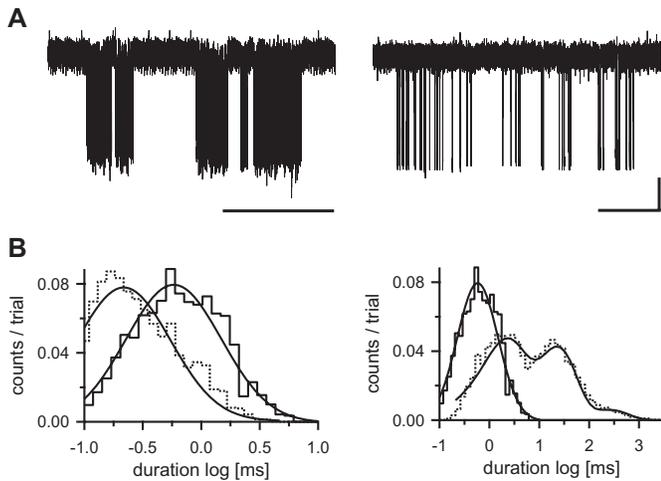
100  $\mu$ M) was applied 300 ms following activation of the nAChR by ACh (100  $\mu$ M; Figure 1B). The inhibition of the ACh-induced current by menthol reached its maximal effect within 100 ms upon application, and the inhibition was  $6.3 \pm 4.0\%$  ( $n = 6$ ;  $P < 0.02$ , Figure 1C) and  $10.1 \pm 5.1\%$  ( $n = 14$ ;  $P < 0.001$ ) for 100 and 200  $\mu$ M, respectively. The block was fully reversible upon termination of the 200 ms menthol coapplication (Figure 1B, black trace). In control experiments, where ACh instead of menthol was applied, we did not observe any alteration in the current kinetic during coapplication (Figure 1B, red trace), ruling out a possible pressure artifact induced by the application system. These results suggest that increase in the time period allowed for the interaction between the nAChR, and menthol increases the degree of inhibition of the nAChR by menthol, whereas the reversibility of inhibition decreases. Based on these findings, in all subsequent experiments, we used a 10 s preapplication period for menthol to ensure maximal inhibition and full reversibility. Menthol itself also elicited small inward currents in 84.9% of all tested cells ( $n = 86$ ).

The size of menthol-induced current was on average  $43.8 \pm 7.8$  pA ( $n = 72$ ) and was independent of the applied menthol concentration (2–1000  $\mu$ M, Figures 1D and 2A). Furthermore, the cooling compound icilin, which potently activates TRPM8 receptors and also TRPA1 receptors (McKemy et al. 2002; Story et al. 2003), did not result in activation of membrane currents, suggesting that TRPM8 as well as TRPA1 receptors did not contribute significantly to the menthol-induced currents in the neurons studied (Figure 1D). These currents have not been further investigated as they do not interfere with the observed inhibition of menthol on the nicotine-induced currents (see Discussion).

Determination of the sensitivity of the nAChRs in trigeminal neurons to acetylcholine, epibatidine, and nicotine revealed  $EC_{50}$  values of 75.7, 0.063, and 40.1  $\mu$ M, respectively (data not shown). In the presence of mecamylamine (10  $\mu$ M), currents elicited by 75  $\mu$ M nicotine were inhibited by  $74.2 \pm 10.5\%$  ( $n = 6$ ;  $P < 0.001$ ). To determine the dose dependence of inhibition of the nicotine-induced currents by menthol (Figure 2B), we choose nicotine at the  $EC_{80}$  (75  $\mu$ M). Figure 2A illustrates for 3 different menthol concentrations the currents induced by menthol itself and its inhibitory effect on nicotine-induced currents. Similar to our findings with ACh-induced currents, we did not observe changes in the kinetics of the nicotine-induced currents by menthol (Figure 2A, lower panel). Inhibiting effects of menthol on nAChR mediated currents were observed for concentrations  $\geq 2 \mu$ M, and maximum inhibition of 91% was observed at 500  $\mu$ M. The corresponding dose–response relationship of the menthol inhibition is illustrated in Figure 2B and fit of the data points to a logistic function revealed an  $IC_{50}$  of 111  $\mu$ M and a Hill coefficient of 1.1. More important, there was no correlation between the degree of inhibition of nicotine-induced currents by menthol and the size of the menthol-induced current (data not shown,  $r^2 = 0.04$ ,  $n = 72$ ). In addition, the TRPM8 receptor selective agonist icilin (10  $\mu$ M) had no effect on the (–) nicotine-induced responses ( $n = 6$ ; data not shown).

To further elucidate the mechanism underlying the nAChR inhibition by menthol, we recorded currents through single nAChR in the cell-attached configuration from recombinant human  $\alpha 4\beta 2$  nAChR expressed in HEK tsA201 cells.

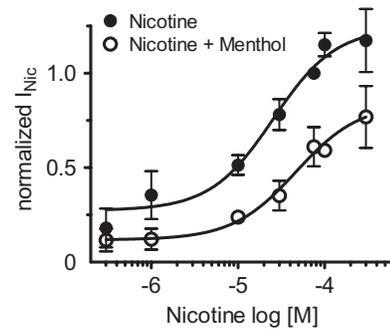
At 100  $\mu$ M nicotine, the openings of the nAChR occurred in clusters, and the pattern of closed intervals in the record was variable (Figure 3A). The open time intervals were described by a single exponential component, whereas closed time intervals were composed of 2 exponential components (Figure 3B). The time constant for the open state was 0.58 ms and was 0.42 and 64.9 ms for the closed state, respectively. In the presence of menthol (100  $\mu$ M), the activity of the nAChR were substantially altered. Channel openings occurred only in brief burst, and the time between bursts was substantially prolonged. For the open state, the time constant was reduced to 0.22 ms, whereas for the closed state, 3 components occurred, with the time constants of 1.44, 19.5, and 295.3 ms,



**Figure 3** Activation of  $\alpha_4\beta_2$  nAChRs by nicotine is modulated in the presence of (–) menthol. **(A)** Individual clusters of nAChRs single channel currents in the presence of 75  $\mu\text{M}$  (–) nicotine (left panel) or in the presence of 75  $\mu\text{M}$  (–) nicotine and 100  $\mu\text{M}$  (–) menthol (right panel). Channel openings are shown as downward deflection. Data are displayed at a bandwidth of 3 kHz. Horizontal and vertical scale bars represent 400 ms and 2 pA, respectively. **(B)** Open (left panel) and close (right panel) dwell-time histograms were constructed from records obtained as in (A). The solid and dotted stair cases represent data obtained with nicotine(–) and nicotine plus menthol, respectively. The smooth curves through the open and closed dwell-time histograms are probability density functions fitted to the data. The time constants and amplitudes for the open state were in ms (%) 0.79 (0.07) and 0.51 (0.076) for nicotine and nicotine plus menthol, respectively. For the closed state, the time constants and amplitudes were 0.68 (0.07), 6.12 (0.005), and 1.17 (0.05), 3.6 (0.028), 4.35 (0.014) for nicotine and nicotine plus menthol, respectively.

respectively. In addition to altering gating properties of the channel, menthol caused 6.1% increase in the amplitude of the single channel current ( $-6.83 \pm 0.91$  vs.  $-7.25 \pm 0.86$  pA;  $n = 5541$  and 3982, respectively,  $P < 0.001$ ).

As the whole-cell current reflects the properties of multiple single channels, our finding on the single channel level strongly suggest that menthol mediates its action not by blocking of the channel pore but rather via an allosteric mechanism reflected in alteration of channel gating properties. To further corroborate this idea, we determined the sensitivity of these receptors and found an  $\text{EC}_{50}$  value for (–) nicotine of 25.5  $\mu\text{M}$  under control and 46.9  $\mu\text{M}$  in the presence of 120  $\mu\text{M}$  menthol (Figure 4). Although menthol induced no significant shift of the  $\text{EC}_{50}$  value, it caused a significant reduction in the efficacy of the agonist nicotine on these receptors. This is visible as parallel downward shift of the dose–response relationship for nicotine/menthol (Figure 4). In contrast to dose–response relationships for nicotine from native receptors in TG neurons, where we observed no responses in the concentration range below 5  $\mu\text{M}$ , we observed for  $\alpha_4\beta_2$  nAChR stably expressed in HEK tsA201 cells small responses within the concentration range between 0.3 and 1  $\mu\text{M}$  that is reflected in the upward



**Figure 4** The sensitivity of human  $\alpha_4\beta_2$  nAChRs to nicotine is reduced in the presence of (–) menthol. Average concentration–response curves were constructed using peak current amplitudes elicited by (–) nicotine (filled circle) or by (–) nicotine in the presence of menthol (120  $\mu\text{M}$ ; open circles). Each data point represents mean  $\pm$  standard error of the mean from 3 to 6 cells. Responses were normalized to those obtained with 75  $\mu\text{M}$  nicotine. The  $\text{EC}_{50}$  value and Hill slope were obtained by fitting the data points to a logistic equation (see Materials and methods), and the best fit is represented by the line through the data points. The  $\text{EC}_{50}$  value for nicotine and nicotine plus menthol was 25.5 and 46.9  $\mu\text{M}$ , respectively. Both fits revealed a Hill slope of 1.2.

shift of the curve for this concentration range. The latter finding is consistent with the observation that  $\alpha_4\beta_2$  nAChR stably expressed in HEK tsA201 cells may express a second population of  $\alpha_4\beta_2$  nAChR that possess different stoichiometry and higher agonist sensitivity (Nelson et al. 2003).

## Discussion

In this study, we show that nAChRs expressed in sensory neurons are reversibly inhibited by menthol with an  $\text{IC}_{50}$  of 111  $\mu\text{M}$ . Single channel recordings from human  $\alpha_4\beta_2$  nAChR in HEK tsA201 cells revealed that menthol causes an increase in single channel amplitude, a shortening of channel open time and a prolongation of its close time. We conclude that the mechanism underlying the menthol-mediated inhibition of nAChR is due to an allosteric modulation of the nAChR by menthol.

### Mechanisms of menthol's action on nAChR

#### Menthol-induced currents

The menthol-induced currents found in our study differ substantially from those described previously in trigeminal neurons by McKemy et al. (2002). The latter showed robust concentration-dependent menthol-induced currents mediated via TRPM8 receptors in a subset of C-fiber neurons (<15%). As we find a much larger fraction of cells responding to menthol (86%), one explanation might be the higher sensitivity of the electrophysiology assay used in our study compared with the intracellular calcium imaging others (McKemy et al. 2002; Peier et al. 2002). However, the observed differences are most likely due to the differences in cell population studied, as we used larger diameters cell

( $33.4 \pm 14.1 \mu\text{M}$ ,  $n = 124$ ) from A $\delta$ -fiber containing neurons in contrast to the C-fiber containing neurons studied by McKemy et al. (2002) and Peier et al. (2002).

#### *Menthol interacts preferentially with the receptor in its closed state*

The observed inhibitory action of menthol appeared to be dependent on the duration allowed for interaction between the menthol and the nAChR as well as the conformational state of the receptor protein itself. Allowing menthol to interact with nAChR prior to channel opening resulted in an increase of its inhibitory activity on the nAChR by  $\sim 80\%$  (from 37 to 66%). Conversely, when the interaction between menthol and nAChR occurred following channel opening, the efficacy of its inhibitory activity was reduced to 6%. Increasing the menthol concentration from 100 to 200  $\mu\text{M}$  did not result in a further increase of current inhibition. The small degree of inhibition observed with the nAChR in the open conformation is unlikely due to the reduced interaction time between the menthol and the receptor, as saturation of the current inhibition is reached within 60% of the total menthol application time (200 ms, see Figure 1B). These findings suggest that interaction between menthol and nAChR is facilitated if the channel protein is in the closed state conformation. Transition of the nAChR to its open conformation obscures the menthol interaction site, which consequently results in a lower efficacy of menthol on the protein complex.

#### *Menthol inhibits the nAChR by allosteric modulation*

Our results indicate that the effect of menthol does not depend on a competitive antagonism. This is suggested by the finding that the  $\text{EC}_{50}$  values of the dose–response curve for nicotine and nicotine plus menthol, respectively, are not significantly different. On the other hand, the dose–response curve is shifted downward reflecting the reduction of the current amplitude over the whole concentration range. It can be ruled out that menthol acts as competitive antagonist on the nAChR. In this case, one would expect a slowing of activation kinetics of whole-cell currents, which was not observed in our experiments (see Figure 2A).

For noncompetitive inhibition, one can distinguish at least 2 different mechanisms. Menthol could act as pore blocker and sterically interfere with the ion flux through the channel protein. On the other hand, it could bind to an allosteric-binding site outside the pore and influence channel gating properties (Arias et al. 2006). Our observation that in the presence of menthol the single channel amplitude is increased rather than reduced, we rule out the idea of fast-acting pore block as observed with, for example, QX 222 (Neher and Steinbach 1978) or a flicker block (Hille 1992). The observed alteration in gating properties more likely supports the idea that menthol acts as a negative allosteric modulator of the nAChR.

#### **Effect of menthol on other ligand gated channels**

Besides its modulator effect on opioid receptors (Galeotti et al. 2002), menthol has recently been shown to be a specific modulator of ionotropic inhibitory receptors. For example, (+) menthol acts as a positive modulator of recombinant GABA<sub>A</sub> and glycine receptors expressed in *Xenopus* oocytes (Hall et al. 2004). In these cases, the allosteric-binding site for menthol is also a binding site for other pharmacologically active substances such as the anesthetic propofol (Watt et al. 2008). Therefore, it would be of interest to analyze if, for example, propofol, which has some structural similarities with menthol, exerts effects on the nAChR and if it can bind to a common site.

#### **Menthol and nicotine interaction**

The most recent findings by Willis et al. (2011) showed that menthol acts as a broad-spectrum counterirritant as it reduced respiratory irritation response of several respiratory irritants found in tobacco smoke. Their data suggest a role of TRPM8 pathways through which activation of TRPM8 by menthol leads to inhibition of the respiratory irritation response. The mechanism underlying this action is currently unknown. Our data extend the findings by Willis et al. (2011) and show that menthol can act as counterirritant directly at the receptor of a major irritant contained in tobacco smoke, nicotine (Lee et al. 2007).

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